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Distinct requirements for the Rad32^{Mre11} nuclease and Ctp1^{CtIP} in the removal of covalently bound topoisomerase I and II from DNA

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Running title: Roles for MRN and CtIP in topoisomerase removal

Summary

For a cancer cell to resist treatment with drugs that trap topoisomerases covalently on the DNA, the topoisomerase must be removed. In this study we provide the first evidence that the *Schizosaccharomyces pombe* Rad32^{Mre11} nuclease activity is involved in the removal of both Top2 from 5' DNA ends, as well as Top1 from 3' ends *in vivo*. A *ctp1*^{CtIP} deletion is defective for Top2 removal, but over-proficient for Top1 removal, suggesting that Ctp1^{CtIP} plays distinct roles in removing topoisomerases from 5' and 3' DNA ends. Analysis of separation of function mutants suggests that MRN dependent topoisomerase removal contributes significantly to resistance against topoisomerase-trapping drugs. This study has important implications for our understanding of the role of the MRN complex and CtIP in resistance of cells to a clinically important group of anti-cancer drugs.

Introduction

Topoisomerases are able to release torsional stress in the DNA or resolve DNA catenanes through a DNA breakage and rejoining mechanism. During break formation the topoisomerase becomes covalently bound to the DNA. This attachment is normally short-lived and reversible, with the protein being released upon re-ligation of the break (Champoux 2001). Camptothecins (CPTs) and Etoposide derivatives are clinically important anti-cancer drugs which increase the half life of the Top1-DNA and Top2-DNA cleavage complexes, respectively (Baldwin et al. 2005; Pommier 2004). The prolonged presence of these complexes and their associated single (in the case of Top1) or double (in the case of Top2) DNA strand breaks interferes with replication and transcription and induces cell killing. Several pathways contribute to the resistance of cells treated with CPT/Etoposide. The first step involves the removal of the topoisomerase from the DNA, after which the remaining DNA break needs to be repaired. Tdp1 has been implicated in the removal of Top1 associated with stalled transcription machinery, rather than stalled replication forks (Pouliot et al. 1999). Deletions of *tdp1* in both *S. cerevisiae* (Liu et al. 2002) and *S. pombe* (E.H., data not shown) confer only mild CPT sensitivity. Also, human patients carrying a *tdp1* mutation do not show genetic instability or susceptibility to cancer and the pathology is most pronounced in non-dividing cells (El-Khamisy et al. 2005; Takashima et al. 2002). A Tdp1-like activity specific for Top2 removal has not been identified. These data suggest that alternative activities are able to efficiently remove Top1 and/or Top2 from the DNA, maybe specifically in the context of stalled replication forks. These alternative pathways might involve nucleolytic cleavage of the DNA, releasing the topoisomerase together with a short DNA fragment (Connelly et al. 2004).

In this study we provide the first evidence that the nuclease activity provided by the *Schizosaccharomyces pombe* Rad32^{Mre11}/Rad50/Nbs1 (MRN) complex is involved in the

removal of Top2 as well as Top1 from the DNA *in vivo*. We also show that Ctp1^{CtlP} is involved in removing Top2, but inhibits Top1 removal, suggesting that Ctp1^{CtlP} plays distinct roles in removing topoisomerases from 5' and 3' DNA ends.

Results

***rad50S* and the nuclease dead *rad32^{mre11}-D65N* mutants are hypersensitive to topoisomerase poisons, but not to other DNA damaging agents**

The topoisomerase-like protein Spo11 becomes covalently bound to the 5' end of the DNA during meiotic DSB formation. *S. cerevisiae rad50S* mutants are deficient in Spo11 removal, suggesting that the Mre11/Rad50/Nbs1 (MRN) complex is involved in Spo11 removal (Keeney 2001). We previously created an *S. pombe rad50S* mutant (*rad50-K81I*) which has been instrumental in the study of meiotic DSB formation in *S. pombe* (Young et al. 2002). This mutant is temperature sensitive for meiotic spore viability (Farah et al. 2005). In a separate study, we have confirmed this observation and found that the decreased spore viability reflects a temperature sensitive defect in the removal of Rec12^{Spo11} from DNA ends. We also found that *rad50S* is proficient for meiotic recombination downstream of Rec12^{Spo11} removal (at restrictive temperature), which is defective in *rad50Δ* (unpublished data).

Because of the topoisomerase-like properties of Rec12^{Spo11}, we wished to establish if MRN is similarly involved in Top1 and Top2 removal in mitotic cells treated with CPT or TOP-53 (an Etoposide derivative; Utsugi et al. 1996), respectively. The high sensitivity of MRN mutants to topoisomerase inhibitors is similar to that of other recombinational repair mutants (Malik et al. 2004), and it remains unknown if the MRN complex is involved in topoisomerase removal, or if this sensitivity can be attributed solely to defects in downstream DNA repair pathways. We used the unique temperature sensitive separation of function properties of *rad50S* to distinguish between an involvement of the MRN complex in topoisomerase removal versus an involvement in (downstream) repair pathways.

First, we tested the sensitivity of *rad50Δ* and *rad50S* to different DNA damaging agents (Fig. 1a). Whereas *rad50Δ* is sensitive to all agents tested (MMS, γ -irradiation, CPT and TOP-53), *rad50S* is only slightly sensitive to γ -irradiation or MMS. At 25 °C (permissive

temperature), *rad50S* is only slightly sensitive to TOP-53 and CPT, but is extremely sensitive to these drugs at 34 °C (restrictive temperature). This temperature sensitive *rad50S* phenotype mimics the meiotic Rec12^{Spo11} removal defect and suggests that *rad50S* might be defective in both Top2 and Top1 removal, but only slightly deficient for recombinational repair functions downstream of removal.

It has previously been proposed that the Mre11 nuclease activity is responsible for Spo11 removal in *S. cerevisiae* (Moreau et al. 1999). We therefore created the putative nuclease dead *rad32^{mre11}-D65N* mutant. This mutant is the equivalent of the well characterised *S. cerevisiae mre11-D56N* mutant, which has been shown to be deficient for nuclease activity and proficient for MRN complex formation (Krogh et al. 2005). Also in *S. pombe*, the *rad32^{mre11}-D65N* mutant is proficient for MRN complex formation (Nick Rhind, personal communication) and is deficient for Rec12^{Spo11} removal (unpublished data). As Shown in Fig. 1b, the sensitivity of *rad32^{mre11}-D65N* to various DNA damaging agents is identical to that of the *rad50S* mutant (at 34 °C), suggesting that *rad32^{mre11}-D65N* might be similarly defective for Top1/Top2 removal, but remains proficient for downstream recombinational repair functions.

The Rad32^{Mre11} nuclease activity is involved in Top1 and Top2 removal

Based on previously published procedures (Keeney et al. 1997; Shaw et al. 1975), we developed an assay (the DNA-linked protein detection or DLPD assay) to detect the presence of topoisomerases covalently bound to the DNA (see Experimental Procedures). We first assessed covalently bound Top1 levels at different time points after CPT addition, and found that these levels were higher in *rad50Δ* compared to WT, at all time points (see Supplementary Fig. 1). Maximum and comparable levels were found 1, 15 and 30 minutes after CPT addition, whereas at 45 minutes the levels start to decrease (possibly due to

increased cell death and/or decreased transcription or replication after prolonged CPT exposure). To interpret these results, it is important to realise that CPT and TOP-53 don't lock topoisomerases permanently onto the DNA, but only increase the half life of the topoisomerase-DNA covalent complex. The topoisomerase will eventually release itself by completion of the catalytic cycle (Champoux 2001) even in presence of these drugs. Estimates using topoisomerases from other organisms *in vitro* suggest these half lives in presence of drugs might be relatively short (Porter et al. 1989; Osheroff 1989). Therefore, the removal defect in MRN mutants as detected with the DPLD assay is probably an underestimate of the real contribution of the MRN complex to topoisomerase removal.

For subsequent experiments, topoisomerase removal was assessed after 15 minutes of CPT/TOP-53 treatment. We consistently found that the amounts of covalently bound Top2 and Top1 are both increased in *rad50Δ* compared to WT cells (Fig. 2). These results were highly reproducible: every single fraction in a series of three experiments using TOP-53 treated cells and five experiments using CPT treated cells showed higher levels of covalently bound topoisomerases in *rad50Δ* compared to WT cells. We quantified the levels of DNA-linked protein (see Experimental Procedures) and found that the total amount of covalently bound protein in *rad50Δ* cells compared to WT shows a 1.7 ± 0.3 and 1.4 ± 0.2 fold increase for Top2 and Top1, respectively. These increases are statistically significant ($P=0.015$, $N=3$ for Top2; $P = 0.007$, $N=5$ for Top1; Paired Student's t-test). We also assessed the role of the Rad32 ^{mre11} endonuclease activity in Top1 and Top2 removal (Fig. 2). Like *rad50Δ*, the *rad32^{mre11}-D65N* mutant also shows increased levels of covalently bound Top2 (2.0 ± 0.2 fold increase) and Top1 (1.7 ± 0.3 fold increase; Fig. 2). These increases are statistically significant ($P=0.02$, $N=3$ for Top2; $P = 0.02$, $N=4$ for Top1; Paired Student's t-test). These data suggest that the MRN Rad32^{Mre11} nuclease activity is involved in both Top1 and Top2 removal.

***ctp1Δ* is deficient for Top2 removal, but over-proficient for Top1 removal**

Mutants of *sae2* in *S. cerevisiae* have a *rad50S*-like phenotype, in meiosis as well as in mitotic cells (Prinz et al. 1997), and it has been shown that Sae2 is involved in Spo11 removal in meiosis (Neale et al. 2005). Recently, a novel gene, called *ctp1*, was identified in *S. pombe* (Limbo et al. 2007; Akamatsu et al. 2008) which shows homology to *S. cerevisiae sae2* and the mammalian tumour suppressor CtIP (Sartori et al. 2007). CtIP/Ctp1 has been shown to interact with the MRN complex (Sartori et al. 2007) and is involved in DSB end resection (Limbo et al. 2007; Sartori et al. 2007). We have found that Ctp1^{CtIP} is required for Rec12^{Spo11} removal (unpublished data).

Because of these observations, we analysed *ctp1Δ* for its ability to remove covalently bound Top2 and Top1 from the DNA. As shown in Fig. 3a, the sensitivity of *ctp1Δ* to DNA damaging agents is identical to that of *rad50Δ* and the double mutant *rad50Δ ctp1Δ*, while *ctp1Δ* is epistatic to *rad32^{mre11}-D65N*. After TOP-53 treatment *ctp1Δ* shows significantly increased levels of covalently bound Top2 compared to WT (Fig. 3b; 1.9 ± 0.2 fold increase after TOP-53 treatment; $P = 0.002$, $N=3$). Surprisingly, after CPT treatment, *ctp1Δ* shows a significant decrease in covalently bound Top1 compared to WT (2.5 ± 0.7 fold; $P = 0.02$, $N=3$) suggesting that the mutant is over-proficient for Top1 removal. To support this unexpected result using an independent assay, we studied the ability of *pku80Δ* (Ku80 homologue; Miyoshi et al. 2003) to rescue the sensitivity of *rad50Δ* and *ctp1Δ* to CPT. Tomita et al. (2003) showed that the sensitivity of *rad50Δ* cells to various DNA damaging agents is rescued by the simultaneous deletion of *pku70*. As this rescue is dependent on Exo1, it was proposed that in the absence of Rad50 the Ku heterodimer needs to be removed from the DSB end to allow processing of the ends by Exo1 (Tomita et al. 2003). Whereas it has been shown that deletion of *pku80* rescues the CPT sensitivity of *ctp1Δ* (Limbo et al. 2007),

we reasoned that *pku80* deletion is unlikely to rescue *rad50Δ* to the same extent for CPT sensitivity, as the covalently bound Top1 molecules would still block DSB repair in the absence of Pku80. As we predicted (Fig. 3c), the deletion of *pku80* strongly rescues the CPT sensitivity of *ctp1Δ*, whereas *pku80Δ* only very slightly rescues the CPT sensitivity of *rad50Δ*. These results support the conclusion that *rad50Δ* is defective, while *ctp1Δ* is proficient for Top1 removal.

The Top2 nucleolytic release product is absent in *rad32^{mre11}*-D65N and *ctp1Δ*

Nucleolytic processing of topoisomerase covalent complexes is predicted to liberate the protein covalently attached to a short DNA oligonucleotide fragment. Neale et al. (2005) developed a procedure to detect Spo11-oligo complexes released from 5' DNA ends. They also reported a Top2-oligo product in *S. cerevisiae*, but this was not dependent on Etoposide and was not affected by mutations in *rad50* and *sae2* (Neale et al. 2005). The biological significance of this product remains unknown.

We adapted this procedure for *S. pombe* to detect a Top2 release product. As shown in Fig. 4a, we detected two bands. The first (lower) band is equivalent to complexes reported by Neale et al. (2005; see lane labelled "C" in Fig. 4a); it is drug-independent and does not depend on Rad32^{Mre11} nuclease activity or Ctp1. We also detected a novel Top2-oligo release product which, as predicted by our previous results, depends on TOP-53 and is undetectable in *rad32^{mre11}*-D65N and *ctp1Δ* mutants. These observations strongly support our conclusion from the DLPD assay, that Rad32^{Mre11} nuclease activity and Ctp1 are involved in nucleolytic removal of covalently bound Top2.

TOP-53 treatment also produces a higher amount of a "smear" signal (Fig. 4), reflecting end-labelling of covalent complexes (rather than a specific release product) that consist of variable lengths of genomic DNA still attached to Top2. In line with our

observations in the DLPD assay, this TOP-53 dependent signal is increased in *rad32^{mre11}*-*D65N* and *ctp1 Δ* compared to WT.

Discussion

Role of the MRN complex in topoisomerase removal

In this study, we provide the first evidence that the Rad32^{Mre11} nuclease activity is involved in Top1 and Top2 removal *in vivo*. It has previously been shown that the bacterial SbcCD complex (Mre11/Rad50 homologues) can remove 5' linked biotin/avidin from oligonucleotides *in vitro* (Connelly et al. 2003). It was also proposed that the MRN complex is involved in removing the adenovirus pTP protein, which is covalently bound to the 5' ends of the adenovirus genome (Stracker et al. 2002). Neale et al. (2005) reported spontaneous Top2-oligo complexes in *S. cerevisiae*, but these were not dependent on MRN, and the cells had not been treated with Etoposide. It thus remains unclear if these complexes are a product of topoisomerase removal. The authors speculated that they might result from Top2 binding adjacent to an existing nick (Neale et al. 2005). In this study we report a novel Top2-oligo species, its dependence on TOP-53 and the Rad32^{Mre11} nuclease activity suggests this is a true release product.

MRN null mutants are among the most CPT/TOP-53 sensitive mutants (Malik et al. 2004). This sensitivity is likely due to a combination of defects in both topoisomerase removal and downstream repair. Our data suggest that *rad50S* and *rad32^{mre11}-D65N* are proficient for recombinational repair downstream of topoisomerase removal and thus that the CPT and TOP-53 sensitivity of these mutants reflects defects in topoisomerase removal. The sensitivity of *rad50S* and *rad32^{mre11}-D65N* mutants to these drugs is less than that of MRN null mutants, but remains very significant (see Fig. 1). This suggests that the MRN complex plays a major role in topoisomerase removal in addition to subsequent recombinational repair.

Whereas *rad32^{mre11}-D65N* mutants are highly CPT and TOP-53 sensitive, the topoisomerase removal defect as measured by the DLPD assay is relatively mild. This

apparent discrepancy might be explained by the ability of topoisomerases to complete the catalytic cycle even in presence of drugs. Furthermore, the high CPT/TOP-53 sensitivity of MRN mutants does not necessarily directly reflect the proportion of covalent topoisomerases removed by MRN. A single covalently linked topoisomerase in S-phase is potentially lethal. If the MRN complex is responsible for the removal of covalently bound topoisomerases in the context of a replication fork, this would thus lead to a disproportionally large reduction of viability in MRN mutants.

The identical sensitivities of *rad50S* and *rad32^{mre11}-D65N* to various drugs (at restrictive temperature for *rad50S*) suggest that the Rad32^{Mre11} nuclease activity might be inactive in the *rad50S* mutant and that Rad50 regulates Rad32^{Mre11} nuclease activity. Indeed, based on structural studies, it has been proposed that ATP-driven directional switching of Rad50 controls the Mre11 nuclease activity (Hopfner et al. 2001). Interestingly, the *rad50S* mutation is found in a probable protein interaction site, and, based on structural studies, it has previously been proposed that this site might interact with Sae2 (Hopfner et al. 2000). Sartori et al. (2007) showed that CtIP interacts directly with the MRN complex. This suggests that CtIP/Sae2 (see below) might control the Mre11 nuclease activity through its interaction with Rad50.

Distinct roles of Ctp1 in Top1 versus Top2 removal

In this study we show that *ctp1Δ* has a defect in the removal of Top2. Surprisingly, *ctp1Δ* is over-proficient for Top1 removal. It is worth noting that *S. cerevisiae sae2Δ* phenocopies *rad50S* for its sensitivity to DNA damaging agents (McKee et al. 1997) suggesting it is not involved in all aspects of recombinational DNA repair, whereas *S. pombe ctp1Δ* closely phenocopies *rad50Δ* (see also Limbo et al. 2007; Akamatsu et al. 2008). The fact that *ctp1Δ* is highly sensitive to CPT, while it is over-proficient in removing Top1, suggests that, like

rad50Δ, *ctp1Δ* is defective in the repair of DNA damage downstream of Top1 removal, possibly because of the role of Ctp1^{CtiP} in 5'-3' nucleolytic resection (Limbo et al. 2007).

In the process of recombinational repair, the 5' and 3' ends of a DNA DSB suffer different fates: The 5' end is nucleolytically resected to expose a 3' overhang (Krogh et al. 2004), which must be protected from resection and is used for strand invasion and copy synthesis. Our observation that Ctp1^{CtiP} is needed for removal of 5' linked Top2 from DNA ends, but inhibits removal of 3' linked Top1, might suggest a role for Ctp1^{CtiP} in protecting the 3' DNA ends.

A recent study (Lengsfeld et al. 2007) showed that purified *S. cerevisiae* Sae2 possesses a nuclease activity which cleaves hairpin DNA structures *in vitro*, cooperatively with the MRN complex (called MRX in *S. cerevisiae*). Purified MRX promotes cleavage by enlarging a single strand gap in the DNA opposite the Sae2 cleavage site. Together with our data this might suggest that Ctp1^{CtiP} is ultimately responsible for the removal of 5' linked proteins (Rec12^{Sp011} and Top2), but our data do not support a direct role of Ctp1^{CtiP} in the removal of 3' linked Top1. Interestingly, Sae2 is able to cut at the base of 5' overhang flaps, whereas it cuts 3' flaps not at the base but within the 3' flap region *in vitro* (Lengsfeld et al. 2007). This difference might explain the distinctive effects of a *ctp1* deletion on Top1 versus Top2/Rec12^{Sp011} removal.

Conclusions and outlook

The involvement of the Mre11 endo/exonuclease activity in the processing of clean DSB ends remains enigmatic: its 3'-5' exonuclease activity is inconsistent with the 5'-3' exonuclease activity required to create the 3' ssDNA ends that are used in the subsequent strand invasion reaction (Krogh et al. 2004). In this study we have demonstrated that the Rad32^{Mre11} nuclease activity is involved in the removal of Top2 from 5' DNA ends as well as

Top1 from 3' DNA ends. Our discovery that Ctp1^{CtlP} is involved in the removal of covalently bound Top2 but inhibits Top1 removal shows that the protein plays distinct roles in removing topoisomerases from 5' and 3' DNA ends. Our findings have important implications for our understanding of resistance mechanisms provided by MRN and CtIP to a clinically important group of cancer therapeutic drugs.

Experimental procedures

Yeast strains and techniques

For strain construction and propagation standard genetic methods and media were used (Gutz et al. 1974). Strains used and constructed in this study are listed in Supplementary Table 1.

DNA-linked protein detection assay

We developed this assay (which is similar to the ICE bioassay; (Subramanian et al. 1995) based on previously published procedures (Keeney et al. 1997; Shaw et al. 1975). In short, untreated cells and cells treated for 15 minutes with 50 μ M CPT or 100 μ g/ml TOP-53 were washed and resuspended in lysis buffer (8 M Guanidine HCl; 30 mM Tris, 10 mM EDTA, 1 % Sarcosyl, pH 7.5) and lysed using glass beads. The cell extract was incubated at 70 °C for 15 minutes. After clarification (15' 13.000 RPM in an eppendorf centrifuge), one aliquot of extract was set aside for DNA quantification (see below) while the rest was loaded on a CsCl gradient, consisting of layers with densities of 1.82, 1.72, 1.50 and 1.45 g/ml respectively. The gradients were centrifuged for 24 hours at 30.000 RPM in a Sorvall AH650 rotor to separate the free proteins from the DNA.

To ensure equal DNA loading, the DNA concentration in the extract was measured and this value was used to adjust the volume of the fractions loaded on the slot blot. For this purpose, the aliquots of extract which were set aside for DNA quantification were treated overnight with RNase and the DNA concentration was determined fluorimetrically using PicoGreen (Molecular Probes/Invitrogen detection technologies).

To confirm that our quantification procedure indeed leads to equivalent amounts of total DNA being loaded onto the slot blot, we quantified the amount of DNA in each of the fractions for 8 independent gradients. As can be seen in Supplementary Fig. 2, the total DNA amounts for each gradient (obtained by adding up the amounts of the individual fractions and

multiplying this value with the volume of each fraction loaded on the slot blot) were very similar, the standard deviation being less than 3 % of the average total DNA for these 8 independent gradients.

To detect covalently bound protein in the DNA fractions the membrane was probed with a specific antibody. To detect Top1, we raised and affinity purified an antibody against peptide FSKREDVPIEKLFSK, (9 amino acids downstream of the active tyrosine). Top2-HA was detected using a monoclonal antibody (Santa Cruz sc-7392). The membrane was processed using standard Western blot procedures and visualised using chemiluminescence. Using this procedure, control cultures of a CPT treated *top1::LEU2* strain, or of untagged strains in the case of meiotic or TOP-53 treated cells, only showed slight cross hybridisation with the top 2 fractions (9 and 10) from the CsCl gradient, which contain the free proteins. These fractions do not contain any DNA, are difficult to load on a slot blot as they tend to clog the membrane, and are therefore not loaded for most experiments.

Signals of the different fractions were quantified using ImageJ (<http://rsb.info.nih.gov/ij/>). Using fraction 6 of *rad50Δ* (showing the strongest presence of covalently bound protein), a two-fold dilution series was made, spotted on a membrane, and processed as described above. Signals of this dilution series were used to create a standard curve. For the standard dilution series and the individual experiments, the signal strength of each band was determined and the background signal was subtracted. The standard curve was used to translate the signal strength of the individual fractions into relative protein amounts. To compare the different experiments with each other, the relative protein amounts for the individual bands were standardised against *rad50Δ* fraction 6. For every experiment, total levels of covalently bound protein were calculated for WT and *rad50Δ* by addition of the protein amounts in the single fractions.

In summary, the signal for Topoisomerase recovered in the DNA-containing fractions

of each gradient are added together for comparison between gradients. As the amount of DNA in these pooled fractions is identical between gradients (see Supplementary Fig. 2), the ratio of Topoisomerase in DNA containing fractions between gradients directly reflects the proportion of covalently attached protein.

To test the difference in levels of covalently bound topoisomerases between WT and mutants for statistical significance, the Paired Student's *t*-test was performed (<http://www.physics.csbsju.edu/stats/t-test.html>). Abbreviations used in the text: *N* sample size (number of experiments); *P* probability that the difference is due to chance.

Detection of Top2 release product

200 ml of culture (1×10^7 cells/ml) were treated for 15 minutes with 100 $\mu\text{g/ml}$ TOP-53. After washing and re-suspension in a volume of 10% TCA equal to the volume of the pellet, the suspension was dropped into liquid nitrogen and subsequently lysed under liquid nitrogen in a 6850 Freezer Mill (SPEX CertiPrep). Subsequent steps were performed according to the published protocol (Neale et al. 2005).

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Figure Legends

Figure 1

Temperature sensitivity of *rad50S* to CPT and TOP-53 suggests that the MRN complex is involved in the removal of covalently bound Top1 and Top2 in mitotic cells. a) The *rad50S* mutant is only slightly sensitive to MMS and γ -irradiation, but is temperature sensitive to CPT (Top1 poison) and TOP-53 (Top2 poison). b) Sensitivity of a *rad32^{mrel1}-D65N* putative nuclease dead mutant to various DNA damaging agents is identical to that of *rad50S* at 34 °C.

Figure 2

Representative examples showing increased levels of covalently bound Top1 and Top2 in *rad50Δ* and *rad32^{mrel1}-D65N* compared to WT. The arrow indicates where the top and bottom fractions of the CsCl gradient have been loaded.

Figure 3

a) sensitivity of *ctp1Δ* against various DNA damaging agents is identical to that of *rad50Δ* and the *rad50Δ ctp1Δ* double mutant. *ctp1Δ* is epistatic to *rad32^{mrel1}-D65N*. b) *ctp1Δ* is defective for the removal of Top2, but over-proficient for Top1 removal in mitotic cells. c) In line with results from the DLPD assay, deletion of *pku80* rescues the CPT sensitivity of *ctp1Δ*, but not that of *rad50Δ*.

Figure 4

a) Autoradiograph showing the TOP-53 dependent Top2-oligo release product in WT cells, which is absent in *rad32^{mrel1}-D65N* and *ctp1Δ*. The control lane C shows the drug-independent Top2-oligo product in *S. cerevisiae*. b) Density trace of the autoradiograph presented in panel a. In WT, the TOP-53 dependent release product is visible as a small peak

left of the spontaneous drug-independent release product.

Figure 1 Hartsuiker et al.

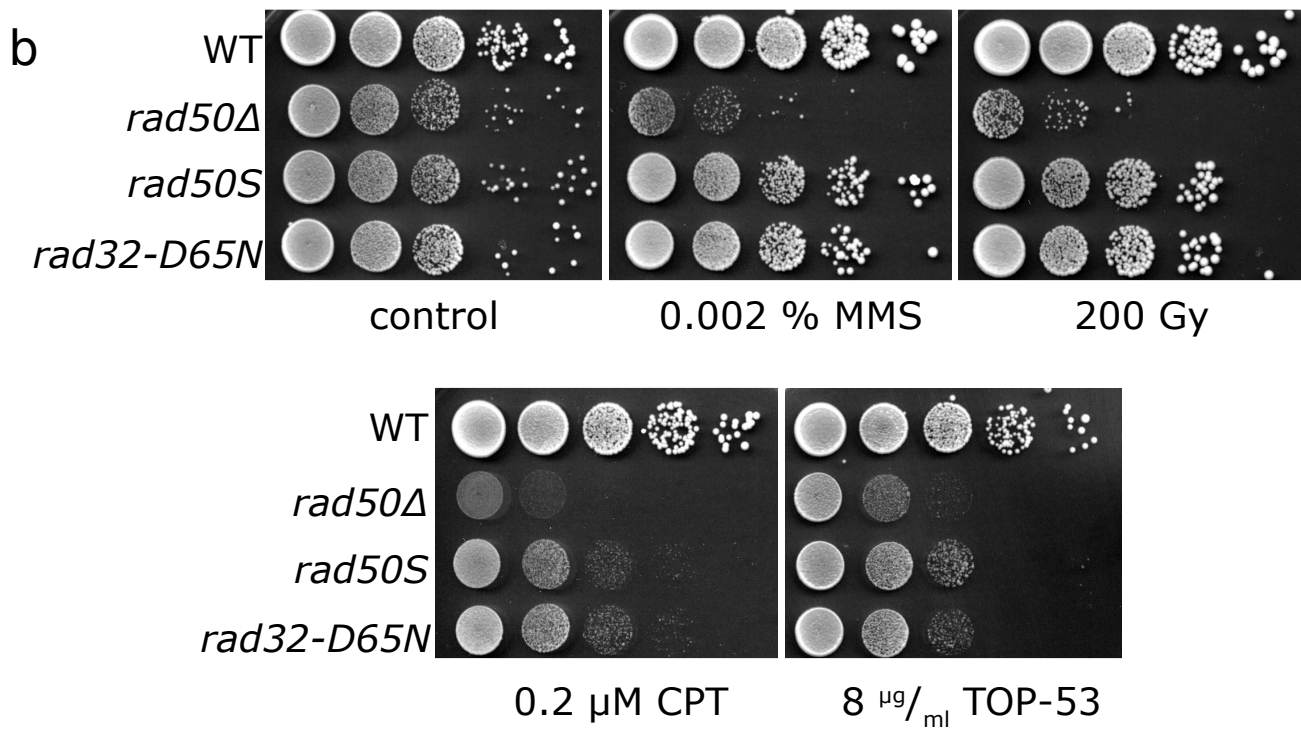
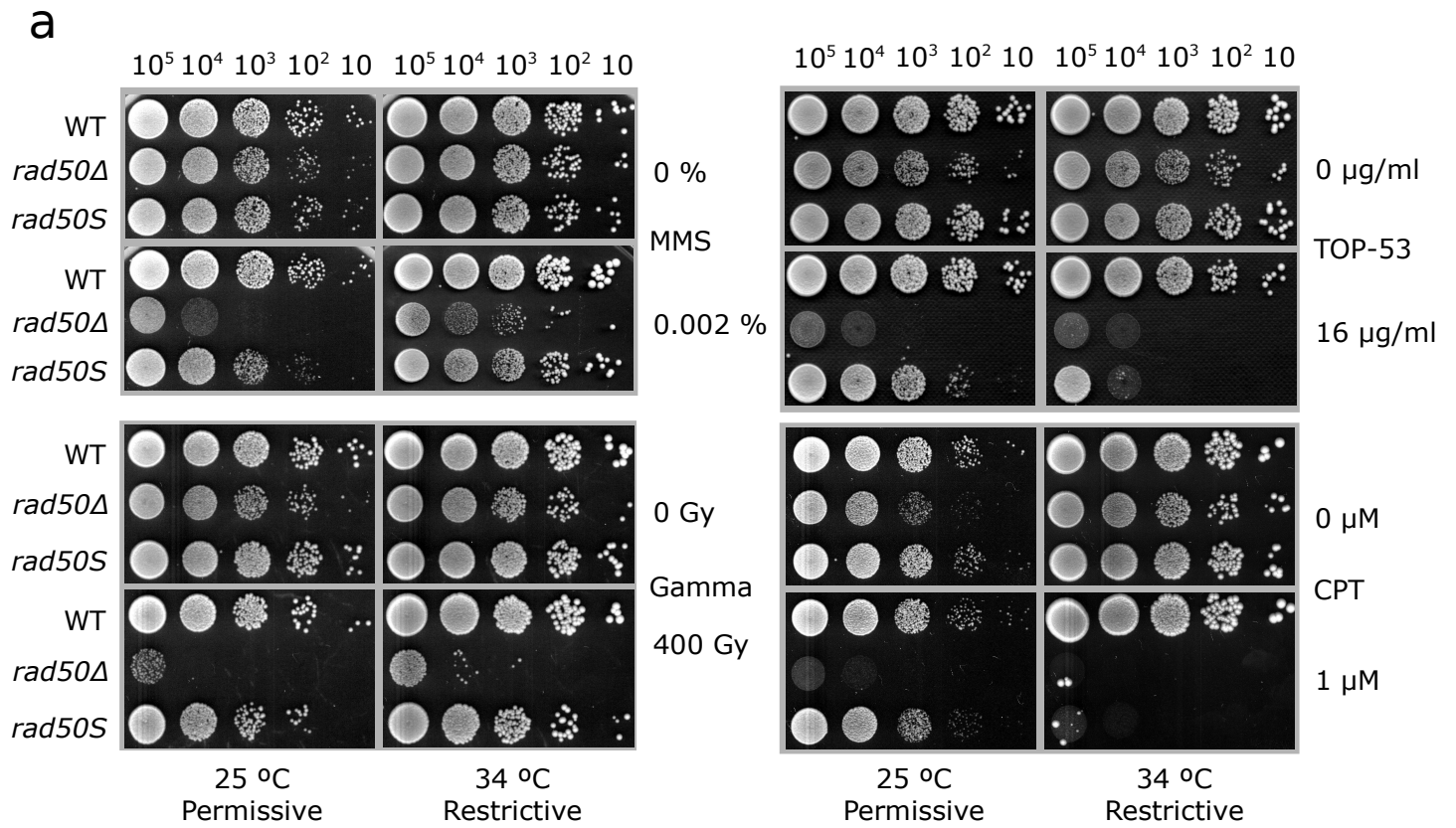


Figure 2 Hartsuiker et al.

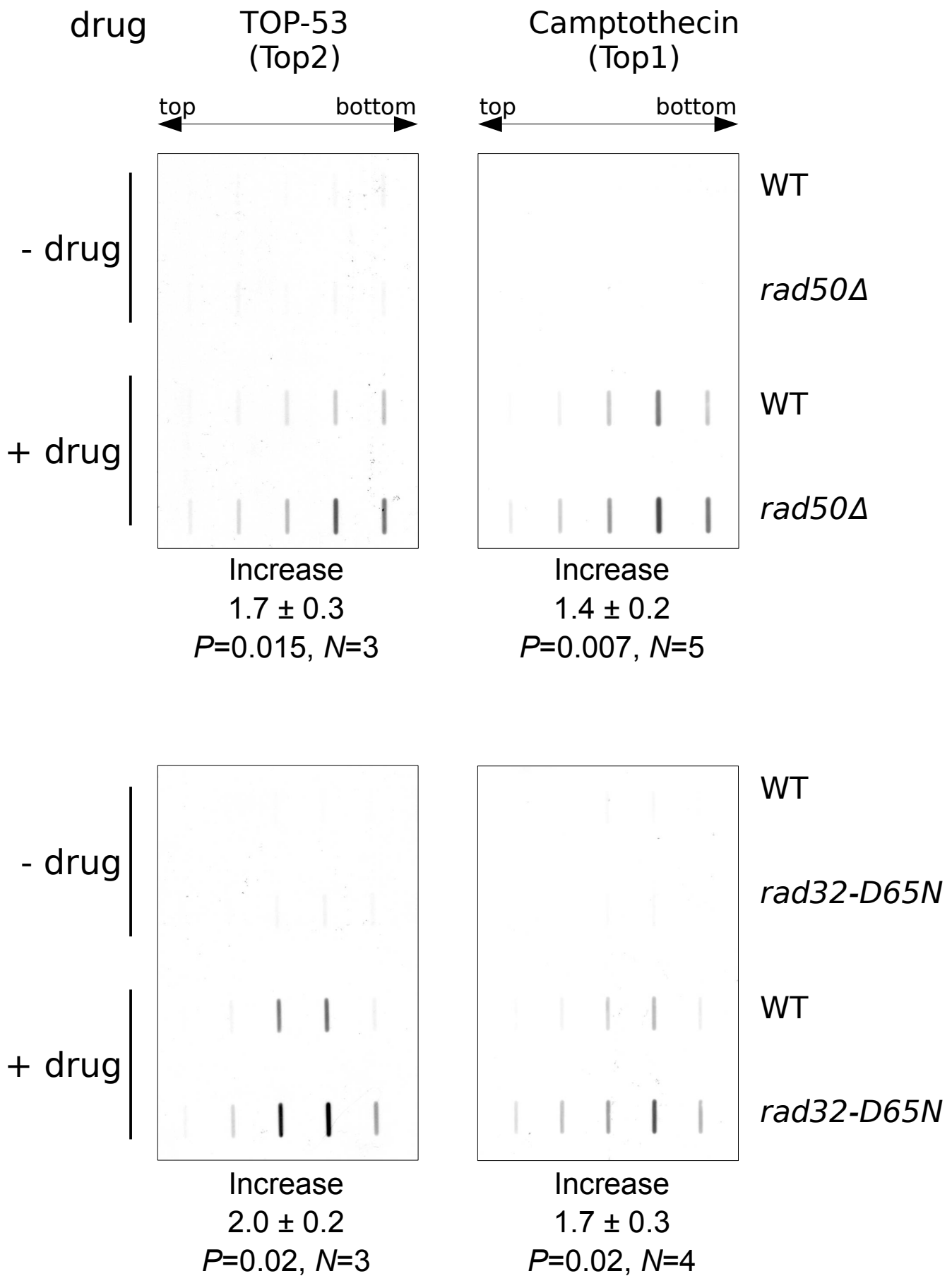


Figure 3 Hartsuiker et al.

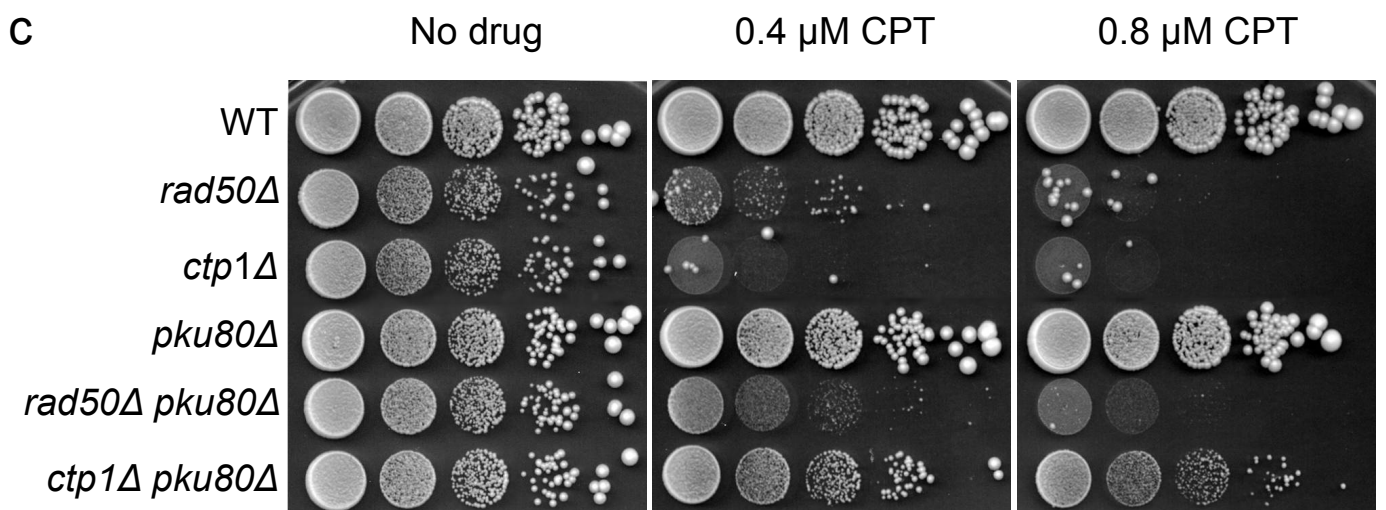
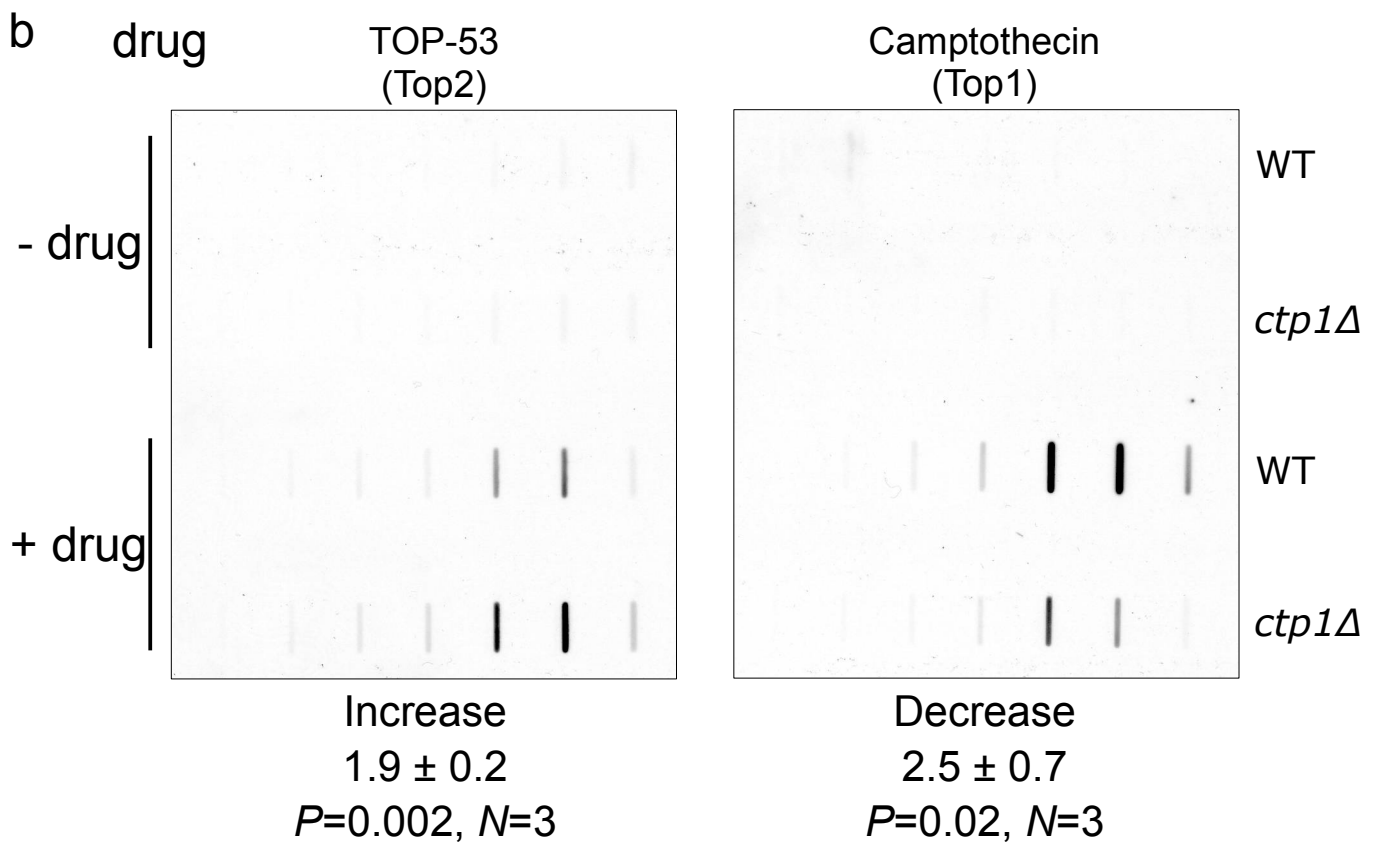
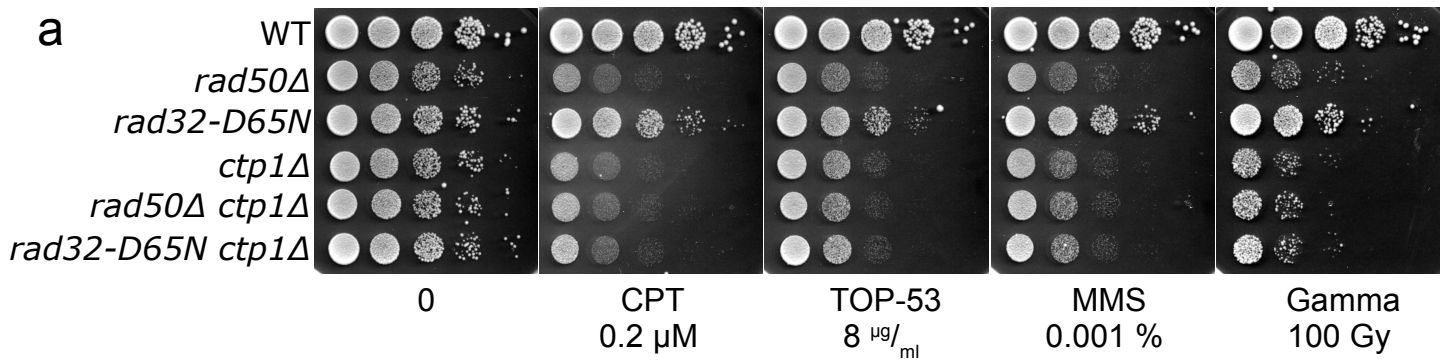
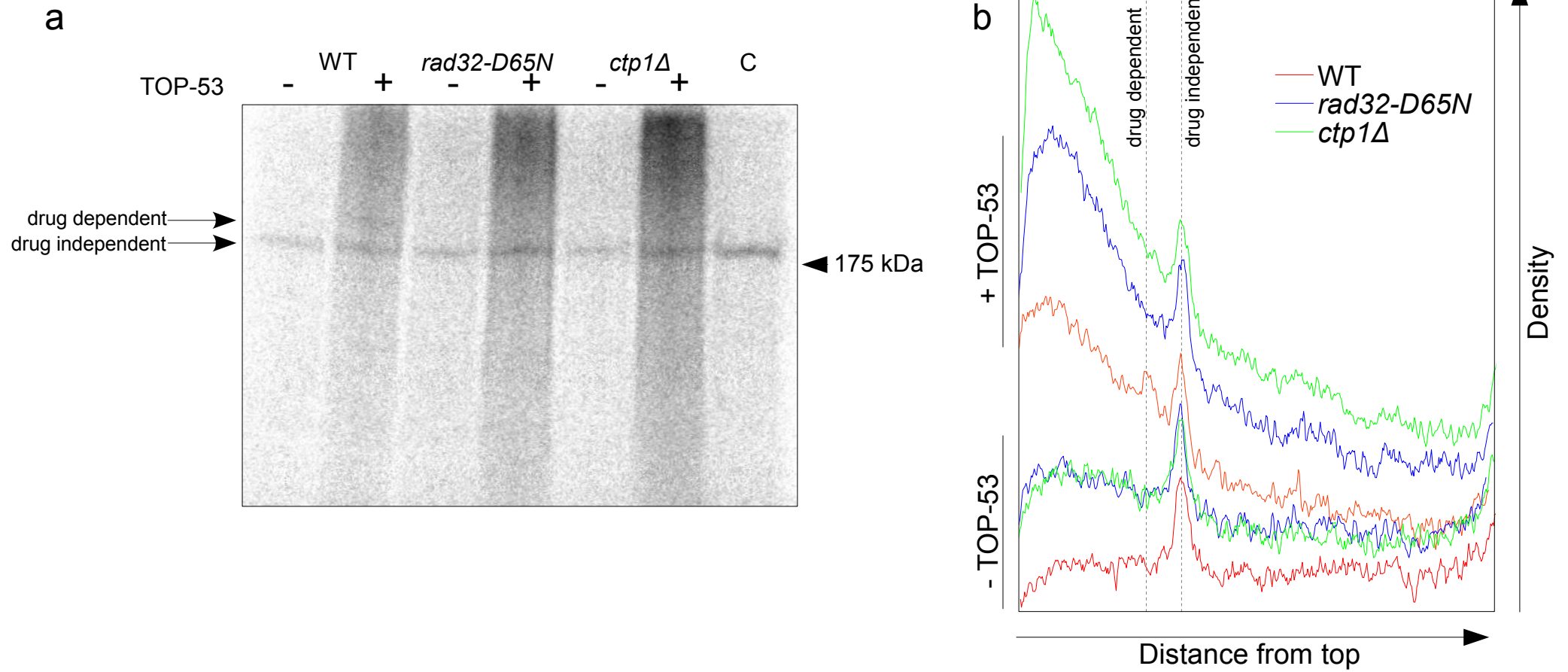
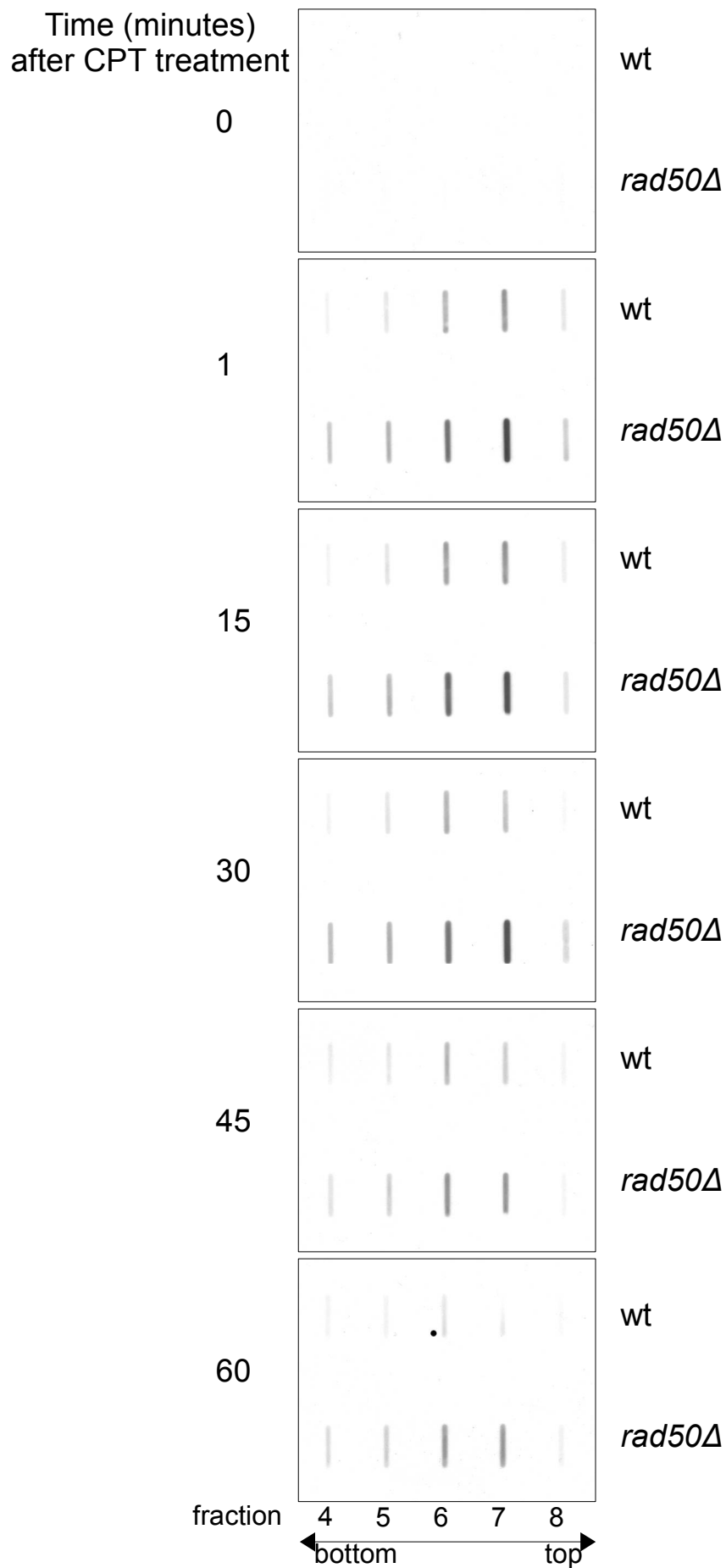


Figure 4 Hartsuiker et al.

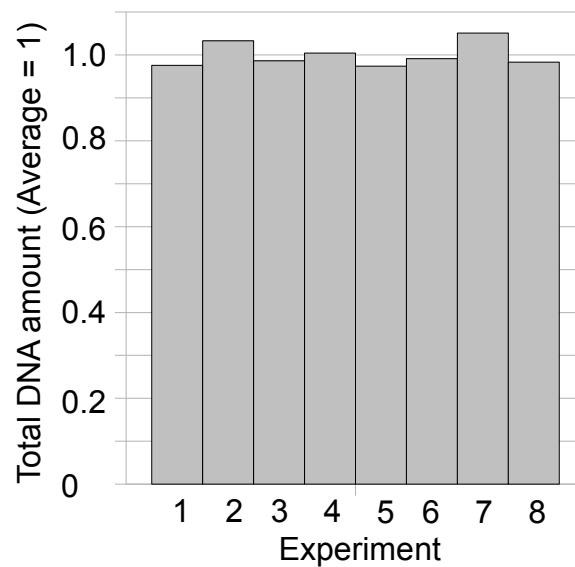


Supplementary Figure 1, Hartsuiker et al.



Levels of covalently bound Top1 in WT and *rad50Δ* at several time points after CPT addition. The arrow indicates where the top and bottom fractions of the CsCl gradient have been loaded. The bulk of the DNA is found in fractions 5, 6 and 7, which show the strongest Top1 signal in *rad50Δ*.

Supplementary Figure 2 Hartsuiker et al.



Quantification of total DNA amounts loaded onto the gradients to verify equal loading. Total amounts of DNA from 8 independent gradients were determined by measuring and adding up the amounts in the individual fractions. Although there was some slight variation of the distribution of the DNA over the different fractions between gradients (data not shown), the total amounts in each gradient were very similar, the standard deviation is less than 3% of the average.